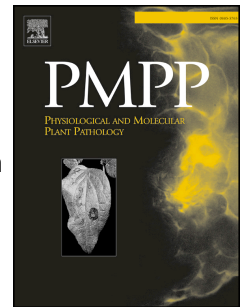


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Infection with blast fungus (*Magnaporthe oryzae*) leads to increased expression of an arabinogalactan-protein epitope in both susceptible and resistant rice cultivars.

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Abstract

Blast fungus (*Magnaporthe oryzae*) is the most serious pathogen of rice. As such, the objective of the current study was to investigate the infection process using a selected panel of anti-plant extracellular matrix antibodies, in infected leaves from three rice cultivars that display varying degrees of resistance to the pathogen; at selected time points post inoculation. An arabinogalactan-protein (AGP) epitope, recognized by the antibody JIM13, was shown to be temporally and spatially regulated during the infection, in all three cultivars. Prior to inoculation, expression of the epitope was confined to the epidermis, sclerenchyma, phloem, xylem and bundle sheath. As the infection progressed, the intensity of labeling increased in all the aforementioned cell types and spread to the mesophyll. Immunogold transmission electron microscopy, revealed this epitope to be most abundant at the plasma membrane. These data suggest that this epitope is borne on a plasma-membrane-associated AGP, which may act as a pattern recognition receptor (PRR), involved in the basal immune response to infection with blast fungus, in both resistant and susceptible rice cultivars. As such, this study represents the first report of a membrane – associated arabinogalactan-protein in rice, which once cloned, could be of utility in future breeding programs/genetic modification to improve the basal immune response to blast fungus and other pathogens, in this globally important food crop.

Keywords

Oryza sativa, *Magnaporthe oryzae*, basal immune response, immunocytochemistry, immunogold labelling.

1. Introduction

Rice (*Oryza sativa*) is the staple food crop for more than 50% of the world's population (Wende *et al*, 2013). In terms of yield losses, the most serious disease of rice is blast caused by the ascomycete fungus *Magnaporthe oryzae*. The annual rice yield destroyed by this fungal pathogen could feed 60 million people and as such this disease is a major threat to global food security (Pennisi, 2010). Therefore, due to the global agronomic importance of rice, an improved understanding of the molecular basis of the interaction between *M. oryzae* and its host is of particular significance. Such information could offer the promise of new and durable disease control strategies, and improved breeding methodologies. Furthermore, the interaction between rice and *M. oryzae* is considered to be a model system with which to study plant - pathogen interactions (Yan and Talbot, 2016).

Like animals, plants possess an innate immune system that recognizes and responds to a plethora of potential pathogens (Boller and He, 2009; Dodds and Nathjen 2010; Wende *et al*, 2013). This plant immune response consists of two layers of protection. The first is a generic defense against pathogens which is governed by extracellular, transmembrane receptors termed pattern recognition receptors. By recognizing conserved pathogen-associated molecular patterns (PAMPs), PRRs thereby trigger a relatively weak basal immune response, known as pattern triggered immunity (PTI). The second, more specific and more potent layer of the plant immune response, is based upon highly polymorphic resistance (R) proteins that are activated upon recognition of highly variable pathogen-associated molecules termed avirulence (Avr)

effectors. This effector-triggered immunity (ETI) is a rapid and robust response, often associated with a hypersensitive reaction (HR) to the specific race of pathogen recognized.

In recent years, the utility of using well-characterised, anti-plant cell wall/plasma membrane antibodies to study plant/pathogen interactions, has shown that they can be used to facilitate an array of techniques (e.g. immunocytochemistry and immunogold labeling) which can be applied to better define modulations in plant cell wall and plasma membrane components *in situ* within complex tissues (Xie *et al*, 2011; Nguema-Ona *et al*, 2013; Wu *et al*, 2017).

Amongst the plethora of polysaccharide, glycoprotein and glycolipid components of the plant cell wall and plasma membrane, the importance of members of the hydroxyproline-rich glycoprotein (HRGP) superfamily, has been shown to be of major significance in plant pathogenesis (Wu *et al*, 2017). Within this superfamily, the potential role(s) of arabinogalactan-proteins (AGPs) is of particular interest. AGPs have been shown to occur both within the plant cell wall and as glycosylphosphatidylinositol (GPI) anchored proteins, located in the plasma membrane and have been demonstrated to play a role in plant – microbe interactions (Xie *et al*, 2011; Nguema-Ona *et al*, 2013; Wu *et al*, 2017).

In light of which, the objective of the current study was to identify plant cell wall and/or plasma membrane-associated molecules involved in the immune response, in rice, when infected by blast fungus. This was performed using an immunocytochemical approach. First, cell wall/plasma membrane epitopes present in

uninfected rice leaves were identified using a selected panel of anti-cell wall/plasma membrane antibodies. Then, the resultant pre-screened panel of antibodies was used to detect developmental regulation of these epitopes during infection by blast fungus of three rice cultivars displaying a range of susceptibility/resistance to the pathogen.

2. Material and methods

2.1 Plant and fungal materials

Three rice varieties, Lijiangxintuanheigu (LTH), International Rice Breeding Laboratory 22 (IRBL22) - which is a monogenic line harboring blast resistance gene Pi-9 - and Yunjing 19 (YJ19) were used in the current study. Cultivar LTH is highly susceptible to infection with blast fungus (*M. oryzae*), cultivar YJ19 is highly resistant to the blast fungus isolate and cultivar IRBL22 displays an intermediate resistance to our test isolate Y98-16t. LTH is recurrent parent of monogenic line IRBL22 and only differs from IRBL22 by the introduction of the resistance gene Pi9. Blast fungus isolate Y98-16t displaying virulence to LTH was selected for use in this study because its virulence and sporulation is well known in our laboratory, and because it is easy to prepare spore-suspensions of this isolate for the inoculation experiments.

2.2 Inoculation of rice leaves with blast fungus

Rice seeds were surface sterilized with sodium hypochlorite (0.2% active chloride) for 5min, washed thoroughly with sterile distilled water and incubated in a wet chamber to germinate at 28°C, in the dark, for 2 days. Germinated seeds were then planted in sterile soil. The rice seedlings were cultivated in a glasshouse facility based at Yunnan Agricultural University at 28°C with a 16h light and 8h dark photoperiod. Once the seedlings had grown to the 3 - 4 leaf stage, the seedlings could be used for inoculation with blast fungus.

The fourth leaf that appeared on the seedlings was used for inoculation. Leaves were inoculated with rice blast spores at a concentration of 3.6×10^4 spores/ml using an adapted version (using leaves rather than leaf sheaths) of an inoculation method previously described by Koga (Koga *et al*, 2004). Circular samples of the inoculated region of the leaf material (3mm in diameter) were then excised at 0hpi, 12hpi, 24hpi, 48hpi and 72hpi for fixation, embedding and subsequent immunocytochemical and immunogold analysis.

2.3 Fixation and embedding of infected leaf material

Inoculated leaf material from (0hpi, 12hpi, 24hpi, 48hpi and 72hpi) was fixed overnight at 4°C, in 4% formaldehyde, 1% glutaraldehyde, 0.1% Triton X-100, 2mM CaCl_2 and 1% sucrose in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10.9 mM Na_2HPO_4 , 1.8 mM KH_2PO_4), pH 7.2. Samples were dehydrated with a series of increasing concentrations of ethanol (10% - 100% v/v). The samples were

embedded in L.R. White resin (London Resin Company Limited, Reading, United Kingdom) at room temperature and allowed to polymerize overnight at 50 °C.

For indirect immunofluorescence light microscopy, 0.9-µm transverse sections of the leaf samples were transferred to polysine-coated glass microscope slides (Agar Scientific, Essex, UK). For immunogold transmission electron microscopy, ultra-thin sections were collected onto formvar/carbon-coated gold grids (200 mesh) (Agar Scientific).

2.4 Antibodies

A total of twenty one rat anti-cell wall/anti – plasma membrane monoclonal antibodies (obtained from Professor Paul Knox, University of Leeds, United Kingdom) which recognized epitopes present on mannans, pectins, AGPs and extensins were used in the current study, to pre-screen leaf material from the three rice cultivars. Eleven of these antibodies were shown to bind to epitopes present in uninfected leaf material and were subsequently used to probe leaves infected with blast fungus. The only antibody that displayed both temporal and spatial regulation of expression in response to infection was JIM13. JIM13 is a rat monoclonal antibody that recognizes an AGP fraction competitive with GlcpUA-B1, -> 3-D-Gal pUA-alpha-1,->2-L-rha trisaccharide (Knox *et al*, 1991; Yates *et al*, 1996).

2.5 Immunolabeling for Light Microscopy

172

173 Sections were blocked in 2% w/v fetal calf serum and 2% w/v bovine serum albumin
174 in PBS, pH 7.2, for 30 min. Samples were incubated with the primary rat monoclonal
175 antibody (JIM13) at 4°C overnight. After being rinsed in blocking buffer 5 times for
176 10 min, sections were incubated with the secondary antibody DyLight 633 conjugated
177 Goat antirat IgG (H + L) (KPL, Gaithersburg, MD, USA) (diluted 1:200) for 1 h at
178 room temperature in total darkness. The sections were extensively washed in blocking
179 buffer (5×10min), rinsed in PBS 8 times followed by sterile distilled water. Sections
180 probed only with the secondary antibody were used as controls. Fluorescence was
181 examined using a laser scanning confocal microscope (Leica TCS SP5 II) at 633nm.

182

183 2.6 Calcofluor white staining

184

185 Sections for light microscopy were stained with 0.01% (w/v) calcofluor white
186 (fluorescent brightener 28, Sigma Chemical, St. Louis, MO) in PBS for 5 min to stain
187 cellulose in order to visualize the cell walls present in the section, and viewed on a
188 laser scanning confocal microscope (Leica TCS SP5 II).

189

190 2.7 Immunolabeling for Transmission Electron Microscopy

191

192 Sections were incubated in blocking buffer (10% w/v bovine serum albumin and 0.1%
193 v/v Triton X-100 in PBS, pH 7.2) for 30 min at room temperature. Sections were then

incubated with primary antibody (JIM13) at 4⁰C overnight and washed extensively by being transferred through 8 drops of blocking buffer. Sections were then treated with 12-nm gold-labeled secondary antisera diluted 1:200 in blocking buffer at room temperature for 1 h. The secondary antibody was a 12nm - gold conjugated goat antirat IgG antisera (obtained from Jackson ImmunoResearch Laboratories). Sections probed only with the secondary antibody were used as controls. Immunogold-labeled sections were stained by 2% w/v aqueous uranyl acetate up to 45 min at 37⁰C followed by Reynolds lead citrate for 7 min at room temperature. Sections were viewed on a JEOL 1200EX transmission electron microscope (TEM) at an accelerating voltage of 80 kV (JEOL, Tokyo).

2.8 Measurement of fluorescence intensity in specific cell types

Fluorescence intensity in various cell types, was measured using a laser scanning confocal microscope (Leica TCS SP5). The software used for this analysis was LAS AF lite 2.4.1 (Leica Application Suite Advanced Fluorescence Lite 2.4.1 Build 6384). Each of the readings presented, is the average fluorescence intensity obtained from three segments of each cell type, comprising of three cells per segment.

2.9 Statistical analysis

The data recorded were compiled in Excel data sheets and subsequently analyzed by the analysis of variance using routines of the statistical software 'SPSS 17' (IBM

SPSS Inc. Chicago, Illinois, USA). The differences between means were compared using the Duncan's New Multiple Range at 5% level of probability ($P < 0.05$). Pearson correlation was used to determine the relationship between data variables and graphs (Figure 3 and Figure 4) were plotted using Sigmaplot 10 .

3. Results

The structure of the rice leaf is illustrated in figure 1. The transverse section stained with Calcofluor white, shows the cell types present, which include upper and lower epidermis, bulliform cells, upper and lower sclerenchyma, phloem, xylem, bundle sheath and mesophyll.

3.1 Immunolocalisation of JIM 13 epitope

Prior to infection with blast fungus (0hpi), indirect immunofluorescence light microscopy revealed that the presence of the AGP epitope recognized by JIM13, is confined to the cells of the upper and lower epidermis, upper and lower sclerenchyma, phloem, bulliform and bundle sheath (Fig. 2A – 2C), in all three rice cultivars. By 12hpi, the JIM13 epitope is also observed to be present at a low level in the mesophyll. At 24hpi, the labelling within the mesophyll has increased slightly relative to 12hpi and by 48hpi the labelling of all cell types is quite distinct. Over time, the intensity of labelling in all cell types gradually increases; so that by 72hpi, all cells present within

the rice leaf section are strongly labelled by the antibody (Fig. 2M – 2O).

When the relative intensity of labelling of the different cell types is compared from 0hpi – 72hpi (Fig. 3), it is clear that the fluorescence intensity at 0hpi increases in the order, mesophyll, bundle sheath, xylem, bulliform, upper epidermis, lower epidermis, phloem, upper and lower sclerenchyma. By 72hpi the order of intensity of fluorescence is xylem, bundle sheath, upper epidermis, lower epidermis, phloem, bulliform, mesophyll, lower, upper sclerenchyma. Over the time course of the experiment, the fluorescence intensity of the lower and upper epidermis, xylem, phloem and lower and upper sclerenchyma cell types remain relatively constant (Fig. 3). Whereas, the intensity of labeling of the bulliform, bundle sheath and mesophyll cells increase over time, with the most marked change being observed in the mesophyll.

From a comparison of the fluorescence intensity of mesophyll cells probed with JIM13 present in the three rice cultivars (LTH, IRBL22 and YJ19) (Fig. 4) it can be seen that the intensity of fluorescence in this cell type, in all three cultivars, increases markedly over time. The fastest rate of increase in intensity of labeling, is observed in LTH followed by IRBL22 then YJ19; with YJ19 displaying the highest fluorescence intensity at 72hpi.

Immunogold labeling confirmed that very little AGP epitope, as recognized by JIM13 was present in the mesophyll cells at 0hpi (Fig. 5). However, by 48hpi the JIM13 AGP epitope was observed to be abundant in the plasma membrane of the mesophyll and by 72hpi a still higher level of expression of the epitope was present (Fig. 5). In

contrast, the plasma membrane of the upper epidermis at 0hpi already contains a significant level of the JIM13 epitope, the level of which remains relatively constant at 48 and 72hpi (Fig. 6).

4. Discussion

Since rice feeds approximately half the world's population and rice blast fungus represents the most serious threat to global rice production, the physiological and molecular basis of the interaction between rice and this pathogen has been the subject of extensive research for many years (Martin-Ulrdiroz *et al*, 2016; Yan and Talbot, 2016). To date, more attention has been focused on the development of the fungal appressorium, penetration and initial infection of the host, ETI and how the fungus manages to suppress plant immunity and rapidly colonize plant tissue, than on PTI and the basal immune response. As such, the data presented are of potential major significance to improve our understanding of the totality of the infection process and the interaction between this agronomically significant pathogen and its host.

Monoclonal antibodies raised against components of the plant extracellular matrix have been shown to provide a powerful 'toolkit' with which to study plant growth and development and stress responses (Roberts, 1989; Knox, 1997; Chen *et al*, 2006; Cornault *et al*, 2015). The use of such antibodies has also been applied to the study of plant/pathogen interactions (Xie *et al*, 2011; Wu *et al*, 2017). The role of plant cell wall hydroxyproline-rich glycoproteins (HRGPs) in the interaction between wax

gourd (*Benincasa hispida*) and the root pathogen *Fusarium oxysporum* in resistant and susceptible cultivars was investigated using a panel of anti-HRGP monoclonal antibodies (Xie *et al*, 2011). This study revealed that extensin epitopes detected by JIM11 and JIM20 were higher in the resistant cultivar in response to infection with the fungus, or after treatment with fusaric acid. These data also demonstrated, that the expression of AGP epitopes as recognized by CCRCM7, was upregulated in the resistant cultivar and that both treatments caused a slight increase in AGP expression in both cultivars as detected by LM2 and JIM16 antibodies. These and other data, indicated that both extensins and AGPs were involved in this host-pathogen interaction and that the CCRCM7 bearing epitope (either AGP or rhamnogalacturonan) most likely contributed to the resistance of wax gourd to the pathogen. Further work on the host response to *Fusarium oxysporum* infection in susceptible and resistant cultivars of banana, has shown that the AGP epitopes recognised by JIM13, JIM8, PN16.4B4 and CCRC-M134 monoclonal antibodies were upregulated in root hairs, xylem and root cap cells of infected plants. In this study, individual AGP and extensin epitopes showed specific radial expression in banana roots. At the transcript level, seven extensins and 23 AGP genes were differentially expressed between the two banana cultivars before and after treatment. These data revealed that AGPs and extensins are involved in wounding and *Fusarium* resistance in banana (Wu *et al*, 2017). These and other recent investigations have linked AGPs to the infectious structures of both beneficial and pathogenic microbes in plant roots (Nguema-Ona *et al*, 2013). It has been proposed that AGPs may contribute to a signaling cascade

responsible for the modulation of the plant immune response (Gaspar *et al*, 2004). Such modulation of the plant immune system upon infection by soil microbes is well documented and contributes to the success or the failure of root infection and disease establishment (Zamioudis and Pieterse, 2012).

As described previously, our data show that the expression of a plasma membrane associated AGP epitope (as recognized by JIM13) is upregulated in rice leaves in response to infection by rice blast fungus in both resistant and susceptible cultivars (Figs. 2 – 6). During the course of the infection the expression of this AGP epitope spreads throughout the leaf, such that by 72hpi all cell types within the leaf are strongly labeled by JIM13.

Based on these data and the fact that the expression of the AGP epitope is upregulated upon infection with blast fungus, irrespective of the level of resistance to the pathogen, we hypothesize that this membrane-associated AGP acts as a component of the basal immune response. Basal immunity/PTI involves the recognition of conserved, indispensable pathogen elicitors termed pathogen – associated molecular patterns by a class of plasma-membrane-associated extracellular receptors termed pattern recognition receptors (Dodds and Rathjen, 2010; Beck *et al*, 2012; Zipfel, 2014). Activation of these PRRs results in active defense responses (Zipfel, 2014), which may contribute to halt infection before the microbe gains a foothold in the plant.

Currently known PRRs, fall into two categories. Firstly, surface-localized receptor kinases (RKs), which have a ligand-binding ectodomain, a single pass transmembrane domain and an intracellular kinase domain and secondly RLPs, which share the same

overall structure, but lack an intracellular kinase domain. Since RLPs do not possess any obvious signaling domains in their short intracellular region, they most likely function in conjunction with one or more RKs to transduce ligand binding into intracellular signaling. We propose that the rice membrane-associated AGP recognized by JIM13 in the current study, acts as a PRR in the form of a RLP. Membrane-associated AGPs do not possess an intracellular kinase domain, but are characterized as β -lectins and this domain could bind to PAMPs or damage-associated molecular patterns (DAMPs) produced by the rice blast fungus during infection.

An arabinogalactan – protein gene family has previously been identified in rice by Ma and colleagues (Ma and Zhao, 2010). In this study, 69 AGPs were identified from the rice genome, including 13 classical AGPs, 15 arabinogalactan (AG) peptides, three non-classical AGPs, three early nodulin-like AGPs, eight non-specific lipid transfer protein-like AGPs, and 27 fascilin-like AGPs. This work was then followed by an in depth study of rice xylogen-like arabinogalactan-proteins (Ma *et al*, 2014). In this study 21 xylogen-like arabinogalactan-protein genes were analysed and their expression during various stages of development was quantified using RT-PCR.

To our knowledge, the current study represents the first use of anti-plant cell wall/plasma membrane antibodies to investigate blast fungus infection of rice and is also the first study to suggest that a plasma membrane-associated AGP may act as a pattern recognition receptor as part of the basal immune response in this interaction. Experiments are currently underway in our laboratory to clone and characterize the

specific rice AGP identified by JIM13; in order to further investigate the role of this membrane-associated glycoprotein in this fascinating and agronomically important plant/pathogen interaction. Moreover, once cloned, this and other PRRs could be used to enable new breeding strategies to enhance PTI in rice and enhance disease resistance in this globally important crop species.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1: General anatomy of rice leaf. Transverse section of rice leaf with Calcofluor white-induced fluorescence showing the cell walls present in this organ. *us* = upper sclerenchyma, *ue* = upper epidermis, *vbs* = vascular bundle sheath, *x* = xylem, *bc* = bulliform cell, *p* = phloem, *ls* = lower sclerenchyma, *le* = lower epidermis, *m* = mesophyll. Scale bar = 20µm.

Figure 2: Immunolocalisation of arabinogalactan-protein containing (JIM13) epitopes via indirect immunofluorescence light microscopy in rice leaves at various time points post inoculation with blast fungus, in three rice cultivars LTH, IRBL22 and YJ19. Transverse sections of the leaf at 0hpi (A, B, C), 12hpi (D, E, F), 24hpi (G, H, I), 48hpi (J, K, L) and 72hpi (M, N, O). *us* = upper sclerenchyma, *ue* = upper epidermis, *vbs* = vascular bundle sheath, *x* = xylem, *bc* = bulliform cell, *p* = phloem, *ls* = lower sclerenchyma, *le* = lower epidermis, *m* = mesophyll. Scale bar = 50µm.

Figure 3: Variation in fluorescence intensity of labeling with JIM13 antibody recorded in different cell types of IRBL22 as affected by time post inoculation with blast fungus. The vertical error bars represent standard error based on three replications.

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492

493

494 Figure 4: Variation in fluorescence intensity of labeling with JIM13 antibody
495 recorded in the mesophyll cells of the IRBL22, LTH and YJ19, at various time points
496 post inoculation with blast fungus. The vertical error bars represent standard error
497 based on three replications.

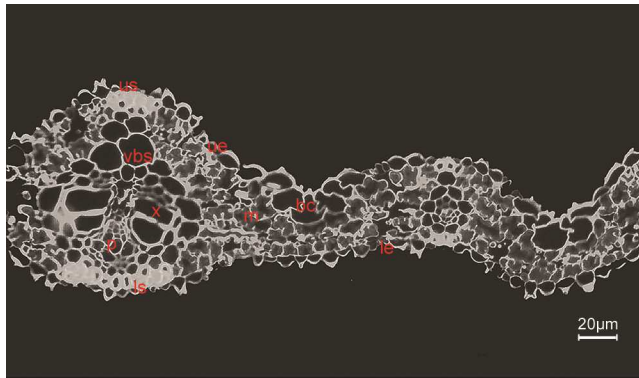
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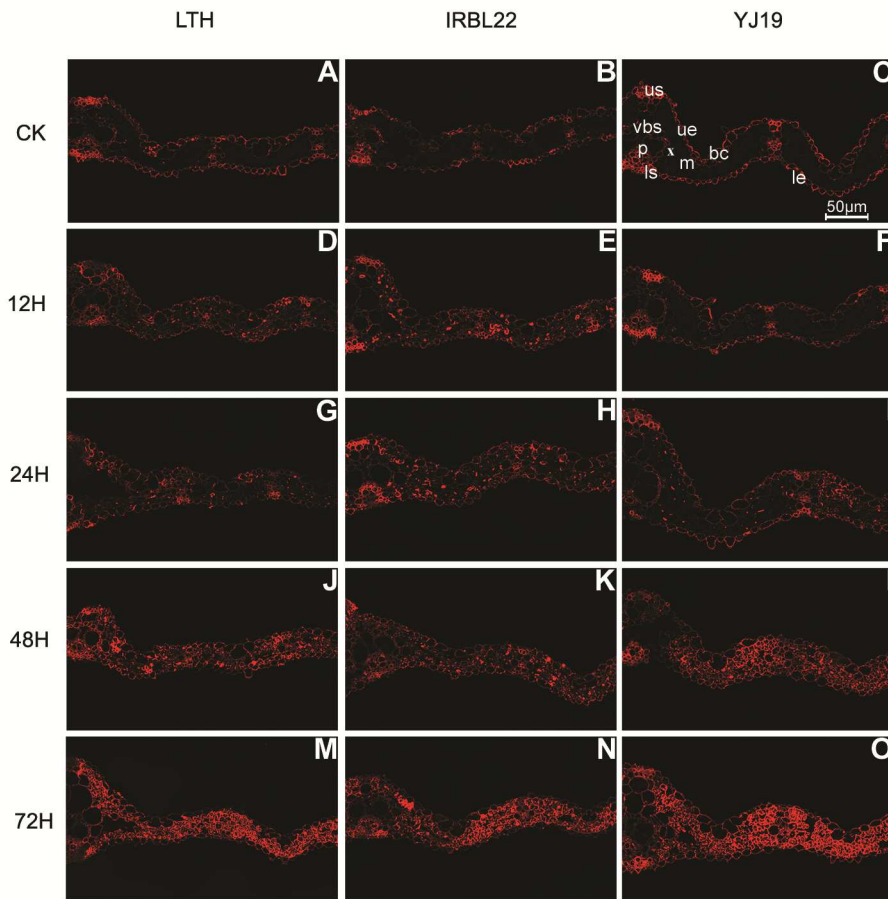
499 Figure 5: Immunogold localization of AGP epitopes using JIM13 in mesophyll cells
500 of IRBL22 at various time points post inoculation with blast fungus. CW = cell wall,
501 ML = middle lamella, PM = plasma membrane, CYT = cytoplasm. Scale bars =
502 0.5 μ m.

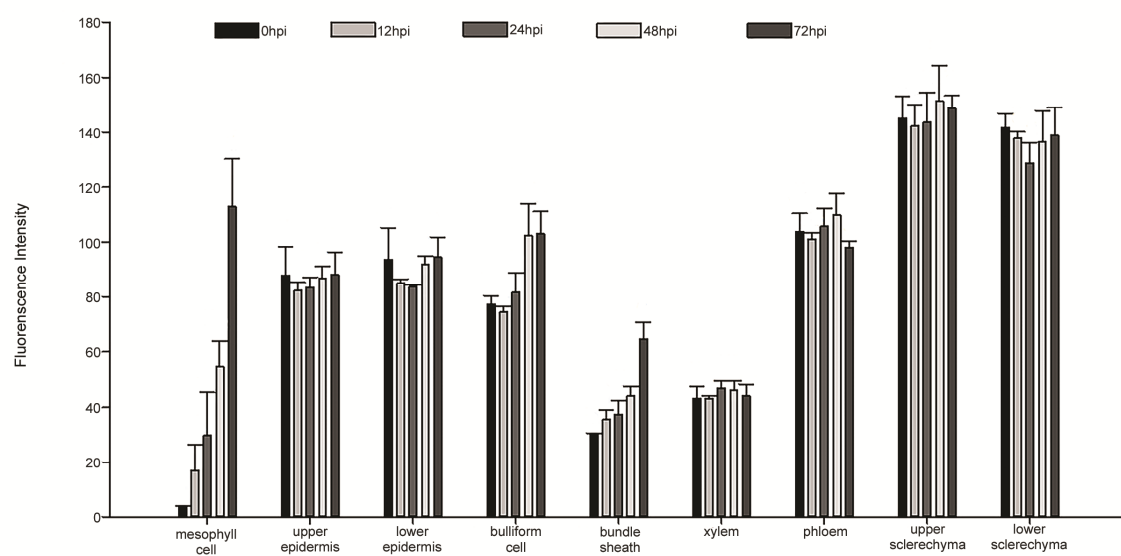
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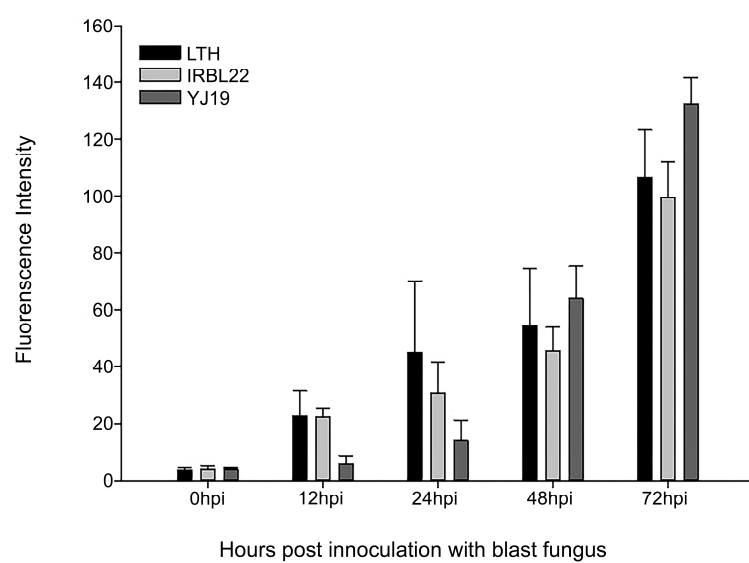
504 Figure 6: Immunogold localization of AGP epitopes using JIM13 in epidermal cells of
505 IRBL22 at various time points post inoculation with blast fungus. CW = cell wall, PM
506 = plasma membrane, CYT = cytoplasm, EP = epidermis. Scale bars = 0.5 μ m.

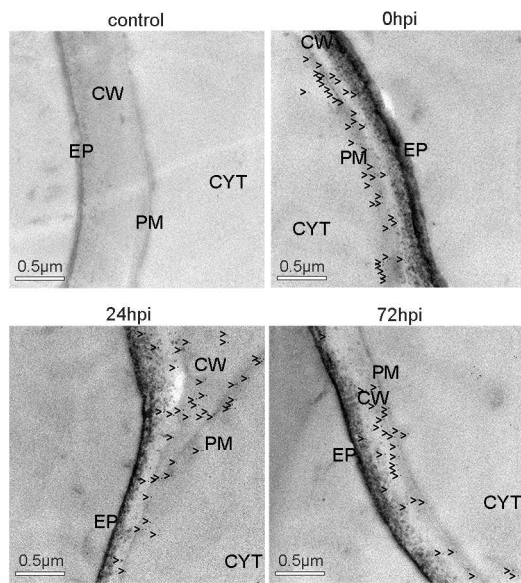
507

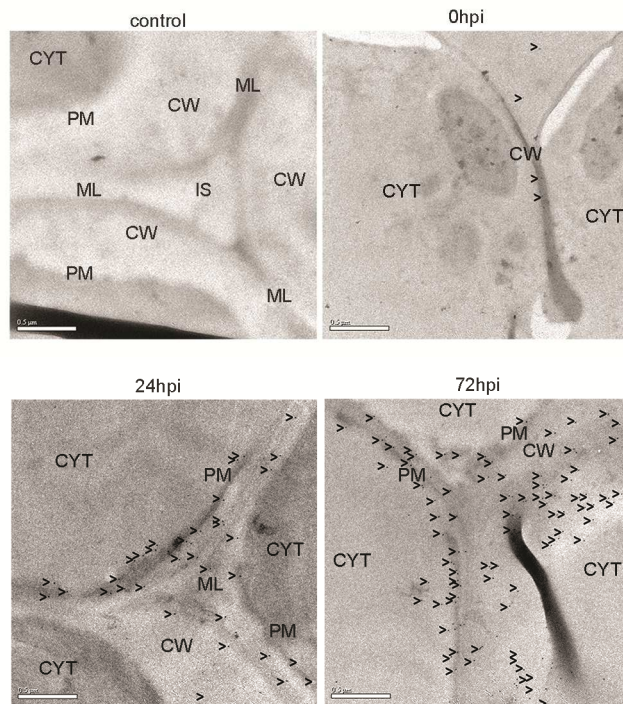












Highlights

- Rice arabinogalactan protein (AGP) epitope detected
- Rice AGP epitope upregulated upon infection with blast fungus
- AGP may act as pattern recognition receptor in basal immune response
- Possible application in future breeding programmes